wherein the lipoprotein is selected from the group consisting HDL, LDL, and VLDL.

Remarks

Amendment to Specification

It is assumed that there was no rejection or objection to the previous amendment to update the priority information nor to amend the title.

The previously submitted abstract has been withdrawn. The new abstract is found almost verbatim in the specification at page 26, l. 20 to page 27, l. 24

Amendments to the Claims

The claims have been amended to delete reference to the processed immunogen as an apolipoprotein. Applicants agree that the claims should be limited to processing of a lipoprotein.

Rejections under 35 U.S.C. 112

Claims 48-51 have been rejected under 35 U.S.C. 112, first paragraph, lack of written description. This rejection is respectfully traversed.

Independent claim 38 defines a method for making antibodies to an epitope of a lipoprotein, wherein the antibody reacts with the lipoprotein independently of lipid content and conformation of the lipoprotein, comprising

immunizing an animal with lipoprotein which has been delipidated, reduced, carboxymethylated, and solubilized with a reducing or denaturing agent, wherein all self-aggregated and degraded material has been removed from the delipidated, reduced, carboxymethylated, and solubilized lipoprotein.

This claim is fully supported by the specification at page 26, l. 20 to page 27, l. 24, and page 28 l. 8-12. See also page 47, lines 1-33, which reads as follows:

"The MAb to Apo B, HB_3cB_3, was produced by immunizing mice with Apo B-100 molecules which had been delipidized, reduced, carboxymethylated, and purified by electrophoresis on a polyacrylamide gel containing 8 M urea. Delipidized Apo B-100 readily precipitates due to self-aggregation in aqueous media. In addition to the self-aggregation, Apo B-100 is also susceptible to fragmentation during the solubilization procedure (Socorro, L. and Camejo, G.J. Lipid Res., 20:631-645 (1979); Olofsson, S.O. et al., Biochemistry, 19:1059-1064 (1980)). **Therefore, in order to separate self-aggregated and degraded material from the preserved protein, the delipidized, reduced, and carboxymethylated Apo B-100 was electrophoresed on a polyacrylamide gel containing 8 M urea.** Coomassie blue staining of the urea-polyacrylamide gel revealed three distinct bands. The most prominently stained band in the urea-containing polyacrylamide gel was cut out immediately after the completion of electrophoresis and subcutaneously injected (while still in the gel) into mice without further manipulation of addition or adjuvants. The most prominently stained band on the urea-polyacrylamide gel had previously been shown to be pure Apo B-100, as confirmed by eluting the band from the urea-containing gel and electrophoresing it under reducing and denaturing conditions on a standard

SDS-containing polyacrylamide gel. The SDS-gel revealed a single protein band of the expected mobility of Apo B-100.

Approximately 10 to 20 µg of the Apo B-100 band excised from the urea-containing gel was injected four times at various locations over a period of two months. The mice immunized with the Apo B-100 according to this procedure were then used in standard methods to produce hybridomas."

This example demonstrates that the antigen was delipidized, reduced, carboxymethylated, and purified by electrophoresis in 8 M urea to remove "self-aggregated and degraded material:. The resulting material yielded three bands – only one having the expected mobility for the apolipoprotein, was cut out and used for immunization. Applicants do not have to presume that the purification step removes self-aggregated and degraded materials – the example states that this was the intended results, and then demonstrated that they achieved this goal.

The argument that "the treatment regime would destroy the lipoprotein as defined by the art" is irrelevant since what is claimed is processing of the lipoprotein to yield an antigen with which to immunize an animal to produce antibody. The examiner's statement that this cannot be done is directly contradicted by the actual example in the application.

As stated at page 47, lines 31-33, the band was eluted from the urea-containing gel and electrophoresed under reducing and denaturing conditions on a standard SDS-gel to confirm that it was pure Apo B-100.

The issues are whether applicants had conception of (1) the subgenus of solubilization

with a reducing or denaturing agent; (2) removal of all self-aggregated and degraded material; (3) soluble lipoprotein as an immunizing material; (4) immunization with an apolipoprotein that is delipidated, reduced, carboxymethylated and solubilized with a reducing or denaturing agent that is free from aggregates and degradation productions and (5) polyclonal antibodies.

The law has long allowed an applicant to claim all that he is entitled to, not forcing him to limit his claims to a specific example, if other means for achieving the same step would be known to those skilled in the art and not require undue experimentation. That is clearly the case here. Once one tells those skilled in the art how to make the antibodies as demonstrated in this application, having the benefits achieved through the method used to create the original antibodies, then anyone could vary the method to achieve the same result, totally circumventing applicant's claims.

Enclosed are copies of pages 5-17, 105-109, and 168-172 of "Immunochemistry in Practice" by Alan Johnstone and Robin Thorpe (Blackwell Scientific Publications 1987). Pages 5-17 describe a variety of ways routinely used in the isolation and purification of proteins, including gel filtration, ion exchange chromatography, and high pressure liquid chromatography (HPLC). Pages 105-109 describe the technique of solubilization of cell membranes and proteins, representative detergents routinely used for such purposes, and criteria to consider in making a selection. Note also the reference on page 105 to "methods employed for soluble proteins with slight modification (e.g. gel filtration, polyacrylamide gel electrophoresis, isoelectric focusing, immunoelectrophoresis and immunodiffusion, immunoassays and affinity chromatography)."

Pages 160-172 describe polyacrylamide gel electrophoresis under denaturing conditions. Note that the SDS is used to denature and solubilize the protein (page 160, section 7.3). See page 169, section 7.3.3, "Most 'insoluble' proteins can be solubilized and electrophoresed in urea." As described at page 169-170, proteins which contain disulfide linked subunits can be split into constituent chains by reduction with dithiothreitol. See also the reference cited in the application at page 27, line 11-13, Lee, et al., Biochim. Biophys. Acta 666:133-146 (1981).

The essential method step to produce antibodies as claimed, is to immunize an animal with the antigen prepared as claimed. This step, with no further action, will cause the animal to make antibodies immunoreactive with the delipidized, reduced, carboxymethylated, and purified to remove self-aggregated and degraded material. These antibodies as they are formed in the animal, are polyclonal. See pages 30-31 of "Immunochemistry in Practice".

These excerpts clearly establish that the immunogen was solubilized, reduced, carboxymethylated, and purified, based on the materials and methods described in the application. Moreover, the examples in the application clearly establish that the solubilized, reduced, carboxymethylated and purified antigen was utilized to elicit numerous antibodies of different specificity in an animal (i.e., polyclonal antibodies), and that spleen cells from the immunized animals could be used to make monoclonal antibodies that could be screened for the desired specificity.

In summary, the methodology which applications used is all well known, as well as the various reagents, substitutions, and variations thereof. What was new, and therefore what the

claims must define to distinguish over the prior art, is that the protein must be delipidized, reduced, carboxymethylated, and purified to remove self-aggregated and degraded material.

Allowance of all claims 48-51, as amended, is earnestly solicited.

Respectfully submitted,

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CERTIFICATE OF UNDER 37 CFR § 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being sent by first class mail with adequate postage to the Assistant Commissioner for Patents, Washington, D.C. 20231 on the date shown below.

Patrea Pabst

Date: March 5, 2002

APPENDIX: Clean Copy of Specification as Amended

Abstract

For the methods and compositions described herein, an antibody is desired that fulfills two important criteria: (i) selective recognition of lipoprotein and (ii) high and invariable reactivity with lipoprotein particles, irrespective of possible variations in their lipid composition and/or conformation. Such an MAb must, therefore, recognize a stable, conformation-independent epitope which is uninfluenced by the lipid content and which is equally expressed in all lipoprotein particles. To obtain an anti-LDL MAb whose binding to LDL particles is not dependent on variations in LDL composition and/or conformation, mice were immunized with delipidized, soluble, reduced, carboxymethylated, and electrophoretically purified Apo B-100. The spleen cells of mice that were immunized using the soluble and electrophoretically purified Apo B, were then used to produce hybridomas according to standard hybridoma methods. A resulting MAb, HB_3cB_3, binds selectively to LDL particles.

APPENDIX: Marked up Copy of the Claims as Amended

48. (four times amended) A method for making antibodies to an epitope of [an apolipoprotein or] a lipoprotein which reacts with the [apolipoprotein or] lipoprotein independently of lipid content and conformation of the [apolipoprotein or] lipoprotein, comprising

immunizing an animal with [apolipoprotein or] lipoprotein which is delipidated, reduced, carboxymethylated, and solubilized with a reducing or denaturing agent, wherein all self-aggregated and degraded material has been removed from the delipidated, reduced, carboxymethylated, and solubilized [apolipoprotein or] lipoprotein.

49. (amended) The method of claim 48 further comprising

isolating the spleen from the immunized animals,

producing hybridomas from the spleen, and

screening the hybridomas for binding to the desired apolipoprotein or lipoprotein.

50. (amended) The method of claim 49 for making antibodies to an apolipoprotein wherein the apolipoprotein is selected from the group consisting of Apo AI, Apo AII, Apo B, Apo CIII, and Apo E.

51. (amended) The method of claim 49 for making antibodies to a lipoprotein wherein the lipoprotein is selected from the group consisting HDL, LDL, and VLDL.

APPENDIX: Clean Copy of the Claims as Amended

48. (four times amended) A method for making antibodies to an epitope of a lipoprotein which reacts with the lipoprotein independently of lipid content and conformation of the lipoprotein, comprising

immunizing an animal with lipoprotein which is delipidated, reduced, carboxymethylated, and solubilized with a reducing or denaturing agent, wherein all self-aggregated and degraded material has been removed from the delipidated, reduced, carboxymethylated, and solubilized lipoprotein.

49. (amended) The method of claim 48 further comprising

isolating the spleen from the immunized animals,

producing hybridomas from the spleen, and

screening the hybridomas for binding to the desired apolipoprotein or lipoprotein.

50. (amended) The method of claim 49 for making antibodies to an apolipoprotein wherein the apolipoprotein is selected from the group consisting of Apo AI, Apo AII, Apo B, Apo CIII, and Apo E.

51. (amended) The method of claim 49 for making antibodies to a lipoprotein wherein the lipoprotein is selected from the group consisting HDL, LDL, and VLDL.

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Notes

1 The standard protein should be structurally similar to that in the unknown solution if possible, because proteins vary somewhat in their colour yield. The standard solution can be aliquoted and stored frozen.
2 The lower limits of detection are about 2 μg in 200 μl and 5 μg in 1 ml; 50 μg in 200 μl has an absorbance of approximately 0.4.
3 Pierce (Appendix 3) have introduced an alternative reagent to Folin-Ciocalteu for detecting the Cu^+ produced by the protein — bicinchonic acid (BCA). This reagent is more stable and does not form precipitates with detergents (see above); the assay is simpler and is claimed to be more sensitive with a broader working range.

1.1.3 Dye-binding assays

Bradford (1976) introduced a protein assay based on the shift in absorbance maximum of Coomassie Brilliant Blue G, from 465 to 595 nm, when it binds to protein. It is simpler and less susceptible to interference by many substances than the Folin phenol method but it does not solubilize filamentous or membrane proteins as well.

The reagent is available commercially (Bio-Rad, Pierce — Appendix 3). Alternatively, dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. Add 100 ml 85% (w/v) phosphoric acid, make up to 1 litre with water and filter through Whatman no. 1 paper (the reagent can be kept for a few weeks, re-filtering if a precipitate forms, but should be discarded after this time; the dye/ethanol stock keeps for years). Add 5 ml of the reagent to 100 μl of test solution (containing 10–100 μg protein), mix, leave for 5 min and read the absorbance at 595 nm. For a more sensitive assay make up the reagent to 200 ml instead of 1 litre and add 0.2–0.8 ml of test solution (containing 1–20 μg protein).

In common with most assays the colour yield varies somewhat between different proteins (see Section 1.1.2, note 1).

An alternative dye-binding assay has been developed recently (Winterbourne, 1986). This measures the amount of dye that binds to protein dried onto filter paper. It is more sensitive than the Bradford or Folin phenol, does not require protein to be in solution and is more specific for protein since small interfering molecules, e.g. peptides, are removed during the washing steps.

Materials and equipment

Protein solution or suspension to be measured
Standard protein (see Section 1.1.2, note 1)
0.4 g Coomassie Brilliant Blue R dissolved in 250 ml ethanol and 630 ml water
Glacial acetic acid
Destain solution: 10% (v/v) ethanol, 5% (v/v) acetic acid
Desorbing solution: 1M potassium acetate in 70% (v/v) ethanol
Whatman 3MM paper
Test tubes to hold 2 ml
Orbital shaker (Luckham — Appendix 3)
Visible light spectrophotometer

Procedure

1 Make a 1 mg/ml solution of the standard protein and calculate the exact concentration from its absorbance at 280 nm (Section 1.1.1).
2 Mark a grid of 1 cm squares in pencil on the paper.
3 In separate squares spot 1, 2, 5 and 8 μl of the standard and up to 8 μl of the test protein solutions. Allow to dry.
4 Mix 6 ml of acetic acid with 44 ml of Coomassie solution and filter. Immerse the paper in the mixture and agitate gently for 1 h.
5 Transfer the paper to destain solution, agitate gently for a few minutes, discard the liquid and repeat until a clear background is obtained (about three times).
6 Dry the paper at room temperature or in an oven.
7 Cut out the squares (including one with no protein) and place in separate test tubes. Add 1 ml of desorbing solution to each, mix, leave for 1 h and read the absorbance of the liquid at 590 nm.
8 Plot the absorbance against protein content for the standard and from this read off the amount of protein in the test solution.

1.2 PROTEIN FRACTIONATION

Two widely used fractionation techniques are described in this section — gel filtration and ion exchange chromatography. Other procedures are considered later (Chapters 7, 9 and 10).

1.2.1 Gel filtration

This is the procedure used most frequently in fractionating proteins. It consists of application of a protein mixture to a column of small beads with pores of carefully controlled size. Large proteins, above the 'exclusion limit' of the gel, cannot enter the pores and so are eluted from the column in the 'void volume' (i.e. the volume of the liquid between the beads; usually this is about 1/3 of the total column volume). Very small molecules enter the pores of the beads fully and have to pass through the total volume of the column before being eluted. Intermediate size proteins partially enter the pores and so are eluted between the void and total volumes in positions logarithmically related to their molecular weight, with some modifications introduced by deviations from a 'normal' globular shape.

Choice of gel. The beads can be carbohydrate or polyacrylamide and are available in a wide variety of pore sizes and hence fractionation ranges (Fig. 1.1). Their method of use is similar, but consult the manufacturer's literature for special considerations.

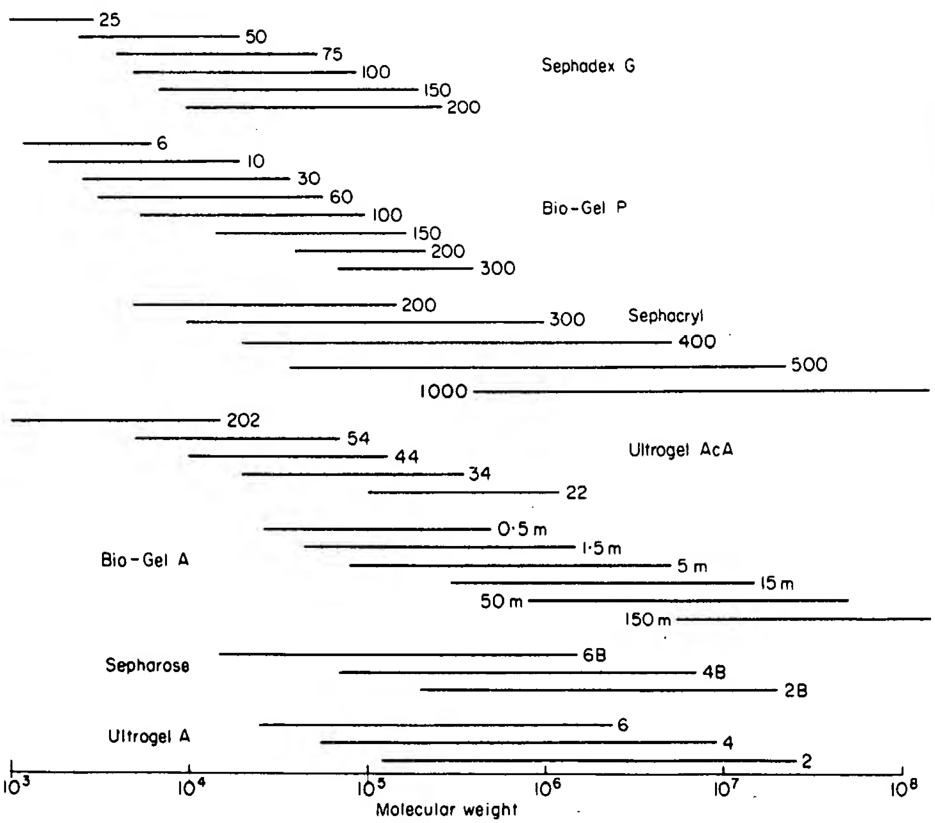


Fig. 1.1 Fractionation ranges of various gel filtration media taken from the manufacturer's literature. The bars indicate the range for which $\log_{10}$ molecular weight is linearly related to elution position (not for superfine grade of Sephadex); the gels are also useful outside these ranges for at least a factor of 2 from each end. Bio-Gel is manufactured by Bio-Rad; Sephadex, Sephacryl and Sepharose by Pharmacia; Ultrogel by LKB (Appendix 3). Sephadex is made from dextran (consisting mainly of glucose); Bio-Gel P from polyacrylamide; Sephacryl from cross-linked dextran; Ultrogel AcA from a mixture of polyacrylamide and agarose; Bio-Gel A, Sepharose and Ultrogel A from agarose (consisting mainly of galactose).

Choice of column. The column used should be of controlled diameter glass (e.g. precision bore tubing, Jencons — Appendix 3) with as low a dead space as possible at the bottom (outflow). Providing these points are considered, home-made columns are perfectly satisfactory except for upward flow applications (see below). Commercial columns are available from several manufacturers (Bio-Rad, LKB, Pharmacia, Whatman — Appendix 3). The tubing connected to the column should be narrow (about 1 mm i.d.) to reduce dead space volume; this is not so important for the top (inflow) connection.

The most useful length of column for gel filtration is 90–100 cm (longer ones or two joined in tandem can be used for greater resolution). The choice of cross-sectional area is governed by the size o[illegible] sample — both volume and amount of protein. The sample volume should not exceed 5% of the total column volume except for desalting (see below, note 7), and resolution is better with less, down to 1–2%. In general 10–30 mg of protein per cm^2 cross-sectional area is a good loading (equivalent to 10–30 mg of protein per 100 ml of gel for a 10[illegible] cm column). More protein will increase the yield but decrease the resolution and hence purity of the product, while lower loading wil[illegible] improve the resolution at the expense of yield.

Cross sectional area $= \pi\ (d/2)^2$
Column volume $= \pi\ (d/2)^2 h$

where d is the internal diameter and h is the length of column.

Packing the column. Ideally an extension reservoir should be attache[illegible] directly to the top of the column so that the total volume of gel an[illegible] buffer can be poured in one and allowed to settle. However, with car[illegible] a column can be packed perfectly well by topping up the gel slurr[illegible] repeatedly as it settles.

1 If the filtration medium is not supplied swollen, equilibrate th[illegible] amount required to fill the column plus 10% with twice the colum[illegible] volume of the buffer (see manufacturer's literature for volume [illegible] swollen gel per unit of dry weight and minimum swelling times).

2 Degas the slurry in a Buchner side-arm flask under vacuum [illegible] water pump with a non-return valve is sufficient) for 1–2 h wi[illegible] periodic swirling. Do not use a magnetic stirrer because this may brea[illegible] the beads.

3 Suspend the gel in about five times its volume of buffer a[illegible]llo[illegible] to stand. When the majority of the beads have settled, remove fin[illegible] (i.e. small particles of beads or other debris) by aspirating the supe[illegible]natant down to 1.5 × settled gel volume.

4 Clean the column with a weak solution of detergent and rinse wi[illegible] water. Clamp the column *vertically* (scaffolding attached to a wall [illegible] best) in an area free from draughts, direct sunlight, heaters a[illegible] vibration.

5 Close the bottom outlet of the column and fill to 10–20 cm heig[illegible] with buffer. Swirl the gel slurry to resuspend it evenly and pour [illegible] down a glass rod onto the inside wall to fill the column (or column a[illegible] reservoir). Allow to settle under gravity for 0.5–1 h to let air bubbl[illegible] escape.

6 Adjust the height of the outlet end of the tube so that the verti[illegible] distance between it and the top of the reservoir or column is less th[illegible]

the maximum operating pressure for the gel (see manufacturer's literature). Unclamp the bottom of the column and allow the gel to pack under this pressure.

7 (a) If an extension reservoir is used, leave the column to pack until the bed just runs dry. Then remove excess gel.

(b) If an extension reservoir is not used, top up the column periodically by syphoning off some excess supernatant, stirring the top of the gel if it has settled completely and then filling the column up to the top with resuspended slurry as in step 5.

8 When the column is packed (gel bed just runs dry) connect the top of the column to a buffer reservoir, remove air bubbles along this length of the tube and allow one column volume of buffer to run through the column. It can be eluted either by the pressure from the reservoir (the operating pressure is the vertical distance between the top of the buffer in the reservoir and the outlet end of the tube; this should never exceed the manufacturer's recommended maximum for the gel), or by a peristaltic pump between the reservoir and top of the column. As a general rule, the flow rate should be the volume contained in 2–4 cm height of column per hour (equivalent to one column volume in 1–3 days for a 90–100 cm long column). Some gels (e.g. Ultrogel and Sephacryl; Fig. 1.1) are designed for more rapid elution without damage to the bed (see manufacturer's literature) but the resolution invariably suffers.

Sample application. If a commercial column is used with flow adaptors touching the top of the gel bed, the sample is most conveniently applied by transferring the inlet tube from the buffer reservoir to the sample, allowing it to enter the tube and gel under operating pressure and then returning the tube to the buffer reservoir. Take care not to introduce air bubbles when the tube is transferred.

For home-made columns without flow adaptors, remove the top of the column (remember to adjust the height of the outlet end of the tube to compensate for the effective height being transferred from the top of the buffer in the reservoir to the top of the column). Either make the sample 5% in sucrose and layer it under the buffer directly to the top of the gel bed or allow the gel to just run dry and apply the sample directly to the top of the gel bed. The sample should be applied carefully by allowing it to run slowly down the inside wall of the column so that the gel bed is not disturbed. When the sample has entered the bed, carefully overlay the gel with buffer and reconnect the top of the column to the buffer reservoir. Check that this disturbance does not allow the gel to run dry during the next few hours of column elution.

Sample elution. For most gels, the maximum flow rate is governed by the operating pressure which the gel can withstand and by the resolution required (see manufacturer's literature and step 8 above). A wide range of fraction collectors are commercially available (Gilson, Isco, LKB, Pharmacia — Appendix 3). Fractions should be collected by measured volume (or counted drops) rather than time to prevent fluctuations in flow rate from altering fraction size. As a general rule, a column volume of eluant from a 90–100 cm long column should be collected in about 100 fractions; the void volume is then eluted at fraction 30–35.

The eluant is usually monitored for its absorbance at 280 nm (proteins) or 215–230 nm (peptides; Section 1.1.1). This can be carried out by reading each collected fraction manually in a spectrophotometer or, more conveniently, by passing the eluant directly through the flow cell of a UV absorbance monitor (Isco, LKB, Pharmacia — Appendix 3). These monitors record transmission (inversely related logarithmically to absorbance) or absorbance at various wavelengths. The older monitors have a fixed wavelength of 254 nm and these detect proteins adequately. In addition radiolabelled proteins or peptides can be detected by taking an aliquot of each fraction for radioactivity measurement (Section 1.4).

Notes

1 The yield of protein should always be greater than 80% and is frequently 90–95%. New gel tends to adsorb protein non-specifically in a saturable fashion, and so the yield improves after the first use (for fractionation of very small amounts of protein the column should be saturated with albumin before use (e.g. Section 5.2.1).

2 Labile proteins can be fractioned at 4°C. Pack the column at room temperature and then transfer it to a cold room and allow it to equilibrate at the lower temperature before applying the sample. Transferring a column from cold to warmer temperatures usually causes bubbles to form and the column must then be repacked.

3 The columns (or swollen gel in a bottle) can be stored at room temperature or lower (do not freeze) in the presence of a bacteriostatic agent (0.02% sodium azide or 0.01% thimerosal). It is advisable to incorporate such an agent in the normal running buffer when columns are used for long periods. Azide interferes with UV absorbance measurements at 230 nm and lower (Section 1.1.1). It also forms potentially explosive lead and copper azide in some plumbing pipes; flush with running water when discarding.

4 For molecular weight determination the column can be calibrated by applying a mixture of standard proteins and noting their elution positions under standard conditions. The large carbohydrate polymer Blue Dextran (Pharmacia — Appendix 3), will mark the void volume of all gel filtration columns.

5 Gel filtration can be performed in a wide variety of buffers. While the majority of separations are carried out in neutral 'physiological' buffers, denaturing conditions are sometimes necessary to separate non-covalently associated polypeptide chains (e.g. Section 3.4.1) or to denature especially elongated proteins for determination of molecular

weight. The separation gels can withstand quite harsh conditions and some are especially strengthened for this purpose (see manufacturer's literature). However, viscous buffers (e.g. guanidine-HCl) reduce the flow rate and tend to cause the gel to compress when the operating pressure is increased. Very harsh denaturing solvents (e.g. 50% formic acid) should be washed out of the column when it is not in use.

6 Commercial columns with flow adaptors can be turned upside down so that the sample and buffer are eluted from bottom to top; all other arrangements (buffer reservoir, outlet, etc.) remain the same. This usually decreases zone broadening and allows a faster flow rate for a given operating pressure because gravity helps decrease the packing of the gel.

7 Gel filtration on small pore gels (e.g. Sephadex G-25, G-50 or other manufacturer's equivalent; Fig. 1.1) can be used to separate proteins from salt, solvent and other small molecules as a quick alternative to dialysis (Section 1.3). It is usually termed desalting. Because the separation is good, much larger sample volumes can be applied (up to 30% of column volume), the overall dilution of sample is small and shorter columns can be used (usually about 30 cm) — see Sections 3.4.1, 5.2.1 and 12.2.3 for examples.

8 To prevent a column without a pump from running dry whilst unattended, arrange the inlet tube so that part of it is below the outlet point of the column.

1.2.2 Ion exchange chromatography

Proteins are bound reversibly to ion exchangers by ionic interactions between oppositely charged groups. The proteins can then be eluted separately by gradually increasing the ionic strength of the buffer, which competitively disrupts the ionic interactions, or by changing the pH so that the interacting groups on the *protein* lose their charge (Fig. 1.2). The pH range is selected to maintain the charge on the *ion exchanger* throughout the procedure otherwise all the bound proteins will be eluted together.

Choice of ion exchanger. Various charged groups on insoluble supports are commercially available and widely used. They are listed together with their usable pH ranges in Table 1.2. Positively charged resins are termed 'anion exchangers' (because they bind and exchange anions); negatively charged resins are termed 'cation exchangers'.

Proteins are amphoteric and their net charge is variable: positive at low pH, negative at high pH and zero at their pI. The interacting charged groups on proteins are mainly carboxyl ($-COOH \rightleftharpoons -COO^- + H^+$) on the one hand and amino or tertiary amino ($-NR_2 + H^+ \rightleftharpoons -NR_2H^+$) on the other. Thus an anion exchanger binds proteins through their unprotonated carboxyl groups and is repelled by

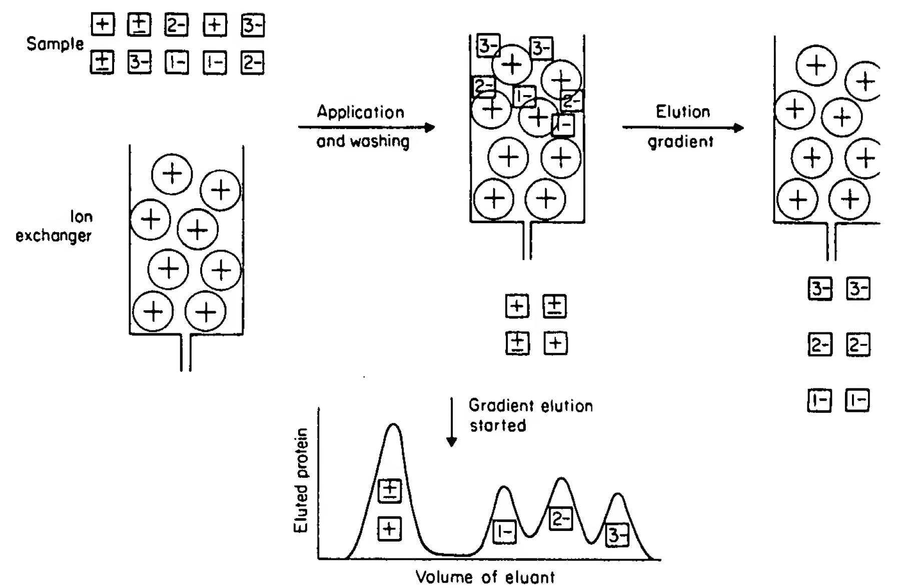


Fig. 1.2 Principle of ion exchange chromatography. The diagram shows the fractionation of variously charged molecules (squares) on an anion exchanger (circles). Positive, uncharged and weakly negative molecules are not bound to the exchanger and elute in the first unretarded peak. More highly charged negative molecules bind to the exchanger and can then be eluted separately (Section 1.2.2): the buffer counter ions have been omitted for clarity but they will bind to all otherwise free charges on both the ion exchanger and sample molecules. For cation exchangers all of the charges are reversed.

Table 1.2 Common ion exchangers

Ionic group	Nominal pH range	Available supports
Anion exchangers		
weak : diethylaminoethyl (DEAE)	2–9	dextran[2] agarose[1,2] beaded cellulose[2] fibrous cellulose[1,3] microgranular cellulose[3]
strong : quaternary amino-ethyl (QAE)	2–10	dextran[2] fibrous cellulose[1]
Cation exchangers		
weak : carboxymethyl (CM)	3–10[a]	dextran[2] agarose[1,2] fibrous cellulose[3]
strong : sulphoethyl (or propyl)	2–12[a]	dextran[2] microgranular cellulose[3]

Manufacturers (Appendix 3): ([1]) Bio-Rad; ([2]) Pharmacia; ([3]) Whatman.
([a]) Pharmacia recommend narrower pH ranges for the dextran and agarose exchangers:

As a general guide, proteins which differ in the total number of aspartic and glutamic residues should be separable by anion exchange while those that differ in their lysine, arginine and histidine content should be separable by cation exchange. This is however not a hard and fast rule because of many other factors which influence the binding. The strength of binding is related both to the protein's pI, and to the total number of charges. Hence, although two proteins with identical pI cannot be separated by equilibrium isoelectric focusing (Sections 7.5 and 9.2.2), they may be separable by ion exchange chromatography using a pH at which they contain significantly different numbers of charged residues per molecule.

Before attempting a novel separation of proteins it is worth electrophoresing them on polyacrylamide gels under both acid and alkaline conditions (Section 7.4). These separations serve roughly as analytical versions of cation and anion exchange chromatography respectively. For example, if a good analytical separation of a mixture is obtained by alkaline electrophoresis then it can probably be separated preparatively by an anion exchanger at a similar pH.

Choice of insoluble support. A variety of insoluble supports for the selected exchange group are available (Table 1.2). These are basically the classical cellulose support and the various beads used for gel filtration (Fig. 1.1).

Cellulose is still favoured overall for protein fractionation although the gel filtration beads are claimed to have a lower non-specific adsorption and are more frequently used for peptide fractionation, especially of small amounts. Some supports, notably Sephadex G-50 derivatives, change their volume alarmingly with changes in ionic strength. The newer physical forms of cellulose, microgranular and beaded (Whatman, Pharmacia — Appendix 3), are claimed to be more reproducible and to have a higher capacity and resolving potential than the older fibrous forms (Whatman, Bio-Rad — Appendix 3). Except when dealing with the most labile of proteins, manufacturer's claims of advantages of high column flow rates are not important because the vast majority of fractionations can be accomplished within 24 h.

A further consideration is the number of charged groups which are present in a unit volume of support (compare manufacturer's values) and which are available to the protein (e.g. Sephadex G-25 derivatives exclude proteins above 5000–10 000 molecular weight from a significant proportion of their charged groups whereas most of the charges on agarose and cellulose derivatives are accessible even to large proteins). This governs the capacity of the column and so will determine the expense, the dilution of sample and loss of protein through non-specific adsorption for a given fractionation. From the manufacturer's literature, the available capacity for protein is very similar (0.11–0.15 g albumin/ml of DEAE derivative) for microgranular cellulose, beaded

Non-specific adsorption to the support is saturable (Section 1.2.1, note 1) and less protein is lost on ion exchangers that have been used and recycled several times.

Preparation of ion exchanger. Some ion exchangers are sold pre-swollen and ready to use. The remainder should be regenerated before use and all exchangers need to be regenerated after each use. The procedure below is a general guide for cellulose exchangers — see manufacturer's literature for specific recommendations. Sephadex and Sepharose derivatives should only be exposed to 0.1 M acid or base.

1 Gently stir the ion exchanger into about 5 × its swollen volume of 0.5 M HCl (anion exchanger) or 0.5 M NaOH (cation exchanger). Leave for 30 min at room temperature with occasional swirling.

2 Filter out the resin by suction through a sintered funnel (porosity 3 or 4) or Whatman no. 54 paper. Wash the resin cake with distilled water until the pH of the filtrate is more than 4 (after acid) or less than 8 (after base).

3 Gently stir the ion exchanger into 5 × its swollen volume of 0.5 M NaOH (anion exchanger) or 0.5 M HCl (cation exchanger). Leave for 30 min at room temperature with occasional swirling.

4 Filter and wash with water as in step 2.

The exchangers have now been washed free of residual protein, and protons or hydroxyl ions left bound weakly to the charged groups of cation or anion exchangers, respectively. These can be displaced easily by the counter ions of the selected buffer when required.

Choice of buffer. The pH should be within the operational range of the ion exchanger (Table 1.2) and also not damaging to the protein. For protein to bind to the exchanger the pH should be at least half and preferably one unit away from its pI (above it for anion exchangers below it for cation exchangers). A pH too far from the protein's pI wi induce excessively strong binding with consequent risks of d ura tion and low recoveries.

A reasonable ionic strength for the application buffer is 0.01–0.05

$$\text{Ionic strength } (I) = \tfrac{1}{2} \Sigma\, c_i z_i^2$$

where c_i is the molar concentration of each ion and z_i is the charge of each ion.

For simple monovalent fully ionized salts the ionic strength is equal the molar concentration:

$$\text{e.g. for 0.1 M NaCl, } I = \frac{0.1 \times 1^2 + 0.1 \times 1^2}{2} = 0.1$$

(for buffer systems usually employed where the pH is between 4 an 10 the concentration of H^+ and OH^- ions is less than 0.1 mM an hence is negligible compared with the concentration of buffer a salts).

For the partially ionized groups of buffer molecules, acting as weak acids ($HA \rightleftharpoons H^+ + A^-$), first calculate the proportion of ionized molecules from the Henderson-Hasselbach equation:

$$pH = pKa + \log_{10} [A^-]/[HA]$$

where [HA] is the concentration of undissociated buffer and $[A^-]$ is the concentration of dissociated buffer.
E.g. 0.01 M sodium phosphate pH 7.0 exists as $Na^+ + HPO_4^{2-} + H_2PO_4^-$ (we can ignore H_3PO_4 and PO_4^{3-} at this pH).
For the reaction $H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$, the $pKa = 7.21$.
Hence $7.0 = 7.21 + \log_{10} [HPO_4^{2-}]/[H_2PO_4^-]$
$\therefore [HPO_4^{2-}]/[H_2PO_4^-] = 0.617$
Total phosphate concentration is 0.01M
$\therefore [HPO_4^{2-}] = 0.00381$ M and
$[H_2PO_4^-] = 0.00619$ M

The concentration of Na^+ balances out these charges
$\therefore [Na^+] = 2 \times 0.00381 + 0.00619 = 0.01381$ M

$$\therefore I = \frac{0.01381 \times 1^2 + 0.00381 \times 2^2 + 0.00619 \times 1^2}{2} = 0.01762$$

For reproducible fractionations the water used in buffers should be as pure as possible. The pH and conductivity of each batch of buffer must be carefully measured and adjusted. Charged bacterial inhibitors such as azide or thimerosal should not be added routinely to buffers (see note 1 below).

Choice of column. See Section 1.2.1 for the physical requirements of a chromatography column. There are even fewer rules for the sample loading of ion exchangers than for gel filtration. As a rough guide, most manufacturers quote a capacity for a standard protein under defined conditions. Allow a safety factor of at least 10 at first and then proceed empirically. In a novel separation check that the column is not overloaded by reapplying the unretarded fraction to a fresh column of regenerated exchanger.

A useful column length for ion exchange chromatography is 20–30 cm, although publication 607 from Whatman (Appendix 3) recommends the use of shorter columns. The volume of sample applied is not important — the binding of proteins is not influenced greatly by their concentration.

Equilibrium of exchanger. The regenerated exchanger should be equilibrated *fully* with the chosen starting buffer before use. The procedure below has been found satisfactory although many alternatives exist. The large variations in ionic strength will adversely affect some supports, notably those based on Sephadex G-50.

1 Add the regenerated exchanger (see above) to an equal volume of $10 \times$ concentration of starting buffer. Mix and leave at room tempera-

2 The exchanger will adsorb some of the buffer ions in exchange for protons or hydroxyl ions and consequently alter the pH. Gently stir the slurry and bring the pH back to the required value using the acid or basic forms of the buffer (e.g. for sodium phosphate buffer use sodium hydroxide for a cation exchanger and phosphoric acid for an anion exchanger).
3 Leave the slurry for a further 30 min, recheck its pH and adjust if necessary. The exchanger is now at the required pH with the required counter ion bound but the ionic strength is too high.
4 Wash the resin on a sintered funnel (porosity 3 or 4) or Whatman no. 54 paper with $5 \times$ volume of starting buffer.
5 Remove the fines and degas the slurry and pack it into the column as described for gel filtration (Section 1.2.1).
6 Wash the column with starting buffer at the operating temperature until the pH and conductivity of the eluant is *exactly* the same as the starting buffer.
The column is now ready for use.

Sample application and elution. The sample should be dialysed exhaustively against the starting buffer; the final volume is not important. It i then applied to the column and the eluant monitored as described fo gel filtration (Section 1.2.1).

After sample application, the exchanger should be washed fir with at least two column volumes of the starting buffer to ensur complete elution of unbound protein. The bound proteins are the eluted by increasing the ionic strength of the buffer or changing its pl (downwards for anion exchangers, upwards for cation exchangers) both. Variations in ionic strength are usually preferred because the can be controlled more carefully. A final ionic strength of 1.0 is usual sufficient to elute most proteins. Either the concentration of the buff can be increased or this parameter kept constant and ot[illegible] ion increased (e.g. NaCl). The latter is usually better because th[illegible]uffe ing capacity, and hence the pH, is constant throughout the separatic procedure.

Except for well-characterized routine procedures, a gradient gradually changing conditions is better than several stepped changes eluting buffer which frequently cause artefactual peaks, although it very difficult to produce a smooth pH gradient. Commercial gradie makers are available (LKB, MSE, Pharmacia — Appendix 3) but th are expensive and generally much more sophisticated than is require Simple apparati for producing linear or concave or convex gradier can be made easily in any laboratory workshop (Fig. 1.3). Rememb that the gradient will not quite reach the conditions in the final buff

As a rough guide, the total volume of the gradient should be 5– times the column volume. The size of fractions of eluant collect should be 1/5–1/10 of the column volume.

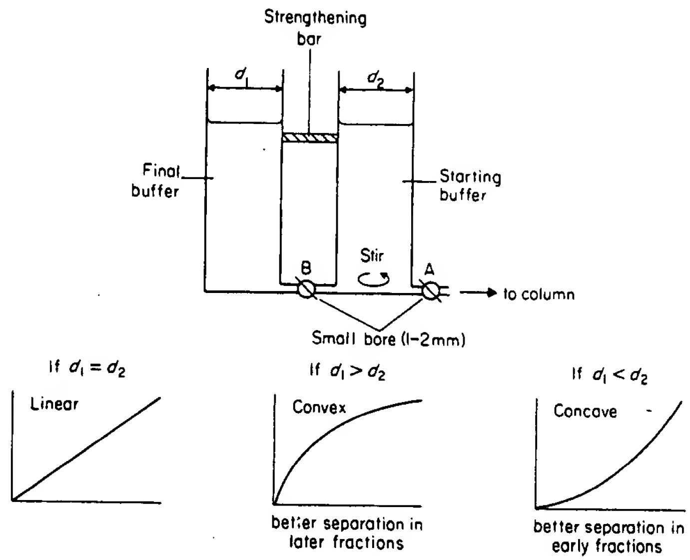


Fig. 1.3 A simple gradient maker. The relative diameters of the glass tubing used to construct the chambers govern the shape of the gradient produced. For convex or concave gradients the ratio should be 1.5–2.0. By adding a third tap so that both chambers have a direct outlet, one apparatus can be used to generate convex or concave gradients depending upon which chamber is chosen for mixing and draw off.

Clamp the apparatus vertically and close both taps. Fill the mixing chamber with starting buffer and flush air from both portions of small bore tubing by briefly opening both taps. Apply sample to the column and with tap A open wash the sample through with starting buffer. Remove excess starting buffer from the second chamber and fill with final buffer until the liquid in the two chambers is *exactly* level, ensuring that air is not introduced into the connecting tubing. Stir the liquid in the mixing chamber vigorously enough to mix to the full height of the liquid column but not enough to cause a vortex. Open tap B and the column will be eluted by the gradient.

Notes

1 Ion exchangers can be stored at 4°C in the presence of 0.03% toluene as a bacterial inhibitor. Alternatively the appropriately charged inhibitor may be used — 0.002% chlorhexidine for anion exchangers; 0.02% sodium azide for cation exchangers.

2 The p*K*a of most charged groups on a protein varies with temperature. Consequently a given separation of proteins will change if repeated at a different temperature. As a further complication, changes in temperature significantly alter the p*K*a of certain 'biological buffers', notably tris, and consequently change the pH of a buffer solution.

1.2.3 Hydroxylapatite

This form of calcium phosphate (also known as hydroxyapatite) has been widely used for fractionation of proteins and nucleic acids. It is a

much lesser extent the phosphate groups interacting with charged groups on the macromolecules. Surface charge density, charge-to-mass ratio and other factors (?) play a role in the strength of adsorption of a protein and provide unique fractionation properties that can often resolve substances inseparable by other methods.

The usual buffer is sodium phosphate pH 6.8 (equimolar Na_2HPO_4 and NaH_2PO_4); most proteins bind in 10 mM and are eluted at 0.5 M. Apply the protein mixture to a column of hydroxylapatite (1 g dry weight for each 2 mg of protein allows a safety factor of about 5) in the low ionic strength buffer and elute with a gradient of increasing molarity (Section 1.2.2). CO_2 can absorb to hydroxylapatite forming a crust on top of the column and so degas all buffers.

1.2.4 HPLC

When these systems were first introduced the initials stood for High Pressure Liquid Chromatography because sophisticated pumps were used to force the material through very fine particles in columns of small cross-sectional area. The technology of the resins has now advanced to such a stage that reasonable flow rates can be obtained without such extreme pressure and 'Performance' is often substituted in the title; Pharmacia call their system FPLC.

Any fractionation of proteins by HPLC uses the same principles as chromatography in standard columns by gel filtration, ion exchange or affinity chromatography (Sections 1.2.1, 1.2.2, 1.2.3, 10.3, *et seq.*). The advantages over the conventional procedures are speed because of the small high-capacity columns, improved reproducibility because of the sophisticated pumps and accurate timers, and in some cases increased resolution because of the fine resins and control systems (e.g. Section 3.4.3, note 1). The disadvantage is the expense of the apparatus and the column materials. All buffers should be degassed and filtered. Systems suitable for protein fractionation are sold by Spectra-Physics, Bio-Rad and Pharmacia (Appendix 3).

1.3 DIALYSIS AND CONCENTRATION OF PROTEINS

If the solvent is volatile (e.g. dilute acetic or propionic acid or ammonium hydroxide or carbonate), a protein solution can be concentrated, to dryness if necessary, simply by evaporation of the frozen solution under vacuum (termed 'freeze-drying' or 'lyophilization'). Organic solvents or inorganic salts can be used to precipitate proteins but this is often selective and also causes some denaturation. Precipitation procedures are useful where the selectivity is advantageous (see for example Section 3.3.1) or the denaturation not important (Section 7.1.5) but they are not used routinely for concentration during general handling of proteins. Chromatography techniques where the protein

2 Production of antibodies

2.1 INTRODUCTION

The extreme specificity shown by individual antibodies for the corresponding antigen makes them ideal tools for the study and purification of proteins. In general, antibodies can be raised easily, even when only a small quantity of immunogen is available, and they are used widely in many biological investigations.

Until recently production of antibodies was limited technically to immunizing animals and, after an appropriate time, collecting the immune serum. Antibodies derived from such immune sera are termed 'conventional antibodies' (Section 2.2). However, Köhler and Milstein (1975) have introduced technology for the production of murine monoclonal antibodies *in vitro* by the construction of hybridomas (Section 2.3). Such reagents have the enormous advantage over conventional antibodies of being homogeneous — i.e. every immunoglobulin molecule is identical in antigen binding properties, allotype, heavy chain subclass, etc. In addition the hybridoma lines secreting the antibodies are immortal and so there is an inexhaustible supply of antibody. More recently techniques have been described for the production of human monoclonal antibodies by viral transformation of B lymphocytes.

Although monoclonal antibodies can be selected to be exquisitely specific they are often of low affinity (Section 6.1.1) and so may be inappropriate for some applications. The possibility of a monoclonal antibody also reacting with other apparently unrelated antigens by virtue of sharing the single small antigenic determinant, or epitope, recognized by the antibody should always be borne in mind.

2.2 CONVENTIONAL ANTIBODY PRODUCTION

Conventional antibody production requires methods for the introduction of immunogen into animals, withdrawal of blood for testing the antibody levels, and finally exsanguination for collection of immune sera.

These apparently simple technical requirements are complicated by the necessity of choosing a suitable species and immunization protocol which will produce a highly immune animal in a short time.

Choice of animal is determined by animal house facilities available, amount of antiserum required (a mouse will afford only 1.0–1.5 ml blood; a goat can provide several litres) and amount of immunogen antigen; goats may require several mg). Another consideration is the phylogenic relationship between the animal from which the immunogen is derived and that used for antibody production. In most cases it is advisable to immunize a species phylogenetically unrelated to the immunogen donor and, for highly conserved mammalian proteins, non-mammals (e.g. chickens) should be used for antibody production. However, if antibodies are to be produced which will detect only subtle differences between proteins (e.g. allotypes) then closely related or even the same species should be used for immunogen isolation and antibody production.

2.2.1 Immunization protocols

A better antibody response is usually obtained by using an adjuvant with the first (priming) immunization. Normally the immunogen is prepared as a water in oil emulsion containing a heat-killed mycobacterium (Freund's complete adjuvant — FCA). The emulsion ensures that the antigen is released slowly into the animal's circulation and the bacteria stimulate the animal's immune system. Further booster (secondary) immunizations are necessary for high antibody levels and these are given either in phosphate buffered saline or as an oil emulsion (bacteria are not normally included in boosting injections; a suitable simple oil adjuvant is Freund's incomplete adjuvant — FIA).

Five routes can be used for injection of immunogen. These are:

(a) intramuscular (i.m.)
(b) intravenous (i.v.)
(c) intradermal (i.d.)
(d) intraperitoneal (i.p.) and
(e) subcutaneous (s.c.)

Any of these can be used for the priming immunization, but i.p. injection is not normally used for large animals, and i.v. immunization cannot be used with particulate antigens or adjuvants. I.d. immunization is very effective in producing a primary response. Immunization by i.d., i.m. or s.c. injection is better in several sites, rather than a single large injection in one site.

Methods for preparing immunogens with Freund's adjuvant and suggested immunization protocols are given in this section.

Preparation of water in oil emulsion for immunization

Materials and equipment

Immunogen in 2 ml phosphate buffered saline (PBS — Appendix 3)
Freund's complete adjuvant (FCA) (primary injection)
or Freund's incomplete adjuvant (FIA) (booster injections) (Difco — Appendix 3)
Emulsifier (Silverson — Appendix 3)

Materials and equipment

Chromic chloride, hydrated
0.15 M sodium chloride (isotonic saline)
Phosphate buffered saline (PBS — Appendix 2)
1 M sodium hydroxide
Erythrocytes — 0.5 ml packed volume
$F(ab')_2$ fragment of antibody — 0.5 mg in 0.5 ml of isotonic saline
Polystyrene conical-bottomed Universal containers 24 mm × 90 mm (Sterilin — Appendix 3)

Procedure

1 Make a 1% (w/v) solution of chromic chloride in isotonic saline and adjust the pH to 5 with sodium hydroxide. Leave at room temperature for 3 weeks adjusting the pH to 5 thrice weekly. The 'aged' solution can be stored at room temperature.
2 Wash the erythrocytes in 3 × 20 ml of isotonic saline by repeated centrifugation (300 *g*, 5 min) and resuspension in a Universal container, discarding the supernatant each time.
3 Resuspend the final cell pellet in isotonic saline to a total volume of 4.5 ml. Add the $F(ab')_2$ fragment in 0.5 ml and mix.
4 Dilute 100 μl of the aged chromic chloride (step 1) in 10 ml of isotonic saline and adjust the pH to 5.
5 Whilst vortexing the cell suspension add 5 ml of 0.01% chromic chloride (step 4) dropwise. Incubate for 15 min at room temperature vortexing every minute for the first 5 min and thereafter every 5 min.
6 Add 10 ml PBS, mix and centrifuge at 300 *g* for 5 min. Discard the supernatant and repeat the resuspension and centrifugation twice.
7 Carry out the rosetting as described in Section 4.3.2, steps 9–12.

Note

A more expensive but better defined alternative is to use small inert hydrophilic beads in place of erythrocytes. These can be purchased with an N-hydroxysuccinimide carboxylate ester group for attachment to proteins (Matrex Pel 102, Amicon — Appendix 3) and even with antibodies of various specificities already coupled (Immunobeads, Bio-Rad — Appendix 3).

4.3.4 Other fractionation techniques

Lymphocytes can also be fractionated using antibodies or lectins coupled to agarose beads instead of erythrocytes (Section 10.3.6). The separation is then by affinity chromatography instead of rosette sedimentation. Alternatively the ligands can be coupled to iron beads which are subsequently removed with a magnet. Both of these separation methods are made more precise by the use of monoclonal anti-

The most reproducible and reliable method for cell fractionation employs fluorescently conjugated monoclonal antibodies to label the lymphocytes of interest which can then be isolated by a flow microfluorimetry (Section 12.2.2). The definition of lymphocyte populations is being improved constantly by this means. Such purified populations should prove of great use in elucidating the relationship between surface markers and lymphocyte function. Unfortunately the expense of the sorting equipment places this method beyond the reach of most laboratories and the simpler procedures described above still have an important role to play. The cell sorter is also very slow for large numbers of cells and antibodies coupled to magnetic beads (Section 10.3.6) are becoming the method of choice.

Centrifugation on Percoll (Pharmacia — Appendix 3) will separate cells by size (see example in Fig. 12.3).

4.4 SOLUBILIZATION OF CELLS AND MEMBRANES

Investigation of the structure of membrane proteins and their interactions with each other and with other proteins has been helped greatly by the use of detergents to solubilize membranes. When present above their 'critical micelle concentration', detergents form micelles which mimic the hydrophobic interior of the lipid bilayer of membranes. This provides a suitable environment for the individual membrane proteins without disturbing their tertiary and quaternary structure (Fig. 4.3). Under the appropriate conditions of detergent excess the membrane structure is disrupted and lipids and proteins are inserted into detergent micelles so that each protein or protein complex is present in a separate micelle. Hence, each behaves as distinct entity and can be studied using methods employed for soluble proteins with slight modification (e.g. gel filtration, polyacrylamide gel electrophoresis, isoelectric focusing, immunoelectrophoresis and immunodiffusion, immunoassays and affinity chromatography).

4.4.1 Choice of detergent

A large number of detergents are available and hundreds of them have been used in biochemical studies (Helenius & Simons, 1975; Helenius *et al.*, 1979). Some of the chemical and physical properties of those most commonly used are presented in Tables 4.3 and 4.4. In general, commercially available detergents are chemically impure, especially non-ionic ones. They contain water or other contaminants in amounts that vary between batches. After prolonged storage of liquid detergents the composition may vary in different parts of the container. In addi-

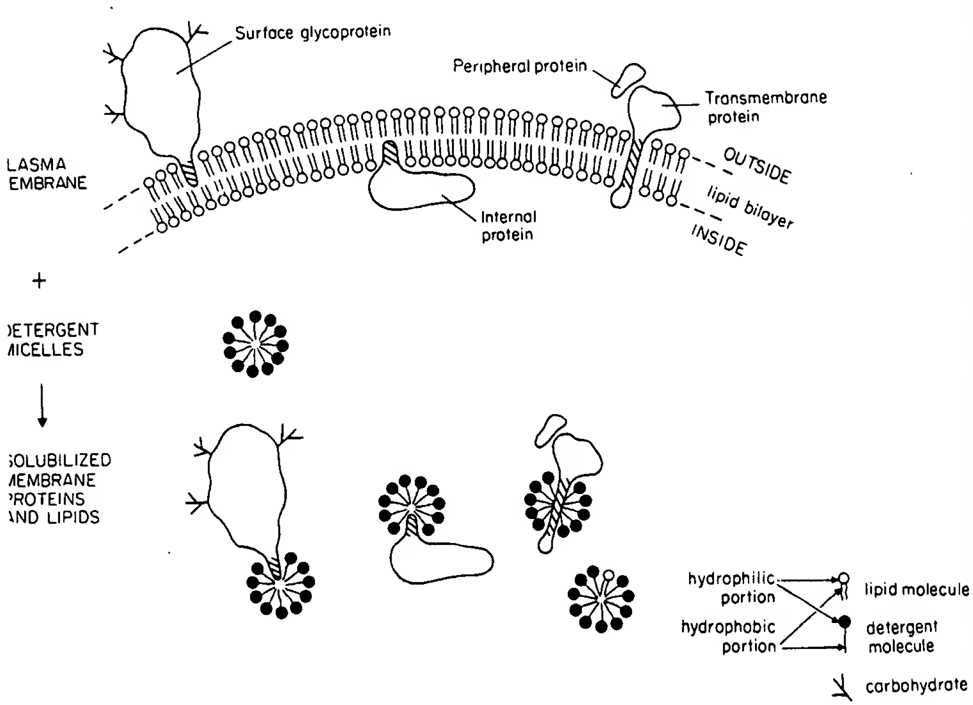
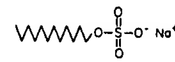
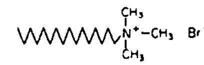
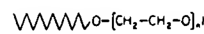
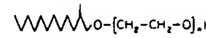
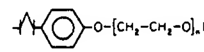
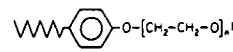
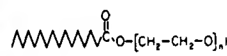
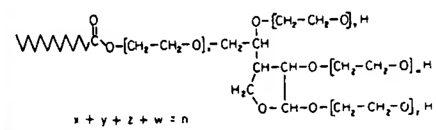
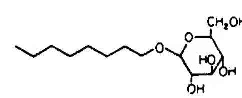
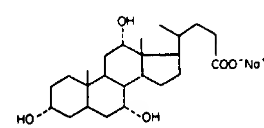
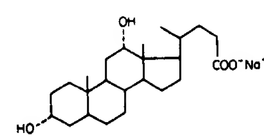
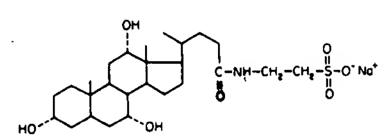


Fig. 4.3 Solubilization of plasma membrane proteins and lipids by detergent. The micelles of detergent mimic the lipid bilayer (see Singer & Nicholson, 1972, for the fluid mosaic model of membrane structure) and provide an environment suitable for the hydrophobic portions (shaded areas) of membrane proteins and lipids.

manufacturer and presented in Table 4.3 represents the majority but not all of the molecules.

Ionic detergents (e.g. sodium dodecyl sulphate, SDS) tend to denature proteins by destroying their secondary, tertiary and quaternary structure, although antibody activity is retained in low concentrations (less than 0.1% SDS). This property is useful for some applications (e.g. polyacrylamide gel electrophoresis, Section 7.3.1) but it does not help in studies of native protein structure. Non-ionic and mildly ionic detergents are less denaturing and can be used to remove a protein from a membrane whilst preserving protein–protein interactions. For example, solubilization of lymphocytes in Nonidet P40 (non-ionic) or plasma membrane in sodium deoxycholate (mildly anionic) releases histocompatibility antigens (termed HLA in humans and H-2 in mice) as two polypeptide chains still held together by non-covalent forces (see Fig. 10.8). In addition, the protein-detergent micelle is recognized by antibodies directed against the antigens (e.g.

Table 4.3 Structure of some common detergents. Modified from Helenius & Simons (1975) with permission

Structural formula	Chemical name	Examples of trade names
	Anionic detergents	
	Sodium dodecylsulphate	
	Cationic detergents	
	Cetyltrimethylammonium bromide	
	Tetradecylammonium bromide	
	Non-ionic surfactants	
	Polyoxyethylene alcohol	Brij series. Lubrol W. AL series
	Polyoxyethylene isoalcohol	Sterox AJ. AP series Emulphogen BC series Renex 30 series
	Polyoxyethylene p-t-octyl phenol	Triton X series Igepal CA series Nonidet P 40
	Polyoxyethylene nonylphenol	Triton N series Igepal CO series Surfonic N series
	Polyoxyethylene esters of fatty acids	Sterox CO series Myrj series Span series
	Polyoxyethylene sorbitol esters[1]	Tween series Emasol series
	β-D-octylglucoside	
	Bile salts	
	Sodium cholate (trihydroxy bile salt)	
	Sodium deoxycholate	
	Sodium taurocholate	

[1] The formula shown is just one molecular type in a complex mixture of different possible structures. n = average number of

Table 4.4 Properties of some common detergents.[1]
Modified from Helenius & Simons (1975) with permission.

Detergent	Commercial name	Aggregation number	Micellar weight ($\times 10^{-3}$)	Critical micelle concentration (mM)
Sodium dodecylsulphate (in H_2O)		62	18	8.2
Sodium dodecylsulphate (in 0.5 M NaCl)		126	36	0.52
Tetradecyltrimethyl ammonium chloride		64	19	4.5
Cetyltrimethyl ammonium bromide		169	62	0.92
POG(4,5) p-t-octylphenol[2]	Triton X-45			0.11
POG(7–8) p-t-octylphenol	Triton X-114			0.20
POG(10) stearyl alcohol	Brij 76			0.03
POG(10) oleyl alcohol	Brij 96			<0.04
POG(10) cetyl alcohol	Brij 56			0.002
POG(9) p-t-octylphenol	Nonidet P40			0.29
POG(9–10) p-t-octylphenol	Triton X-100	140	90	0.24
POG(9–10)nonylphenol	Triton N-101			0.085
POG(10) nonylphenol		100	66	0.075
POG(10) tridecyl alcohol		88	56	0.125
POG(12–13) p-t-octyl-phenol	Triton X-102			0.3–0.4
POG(14) stearyl alcohol		370	330	0.06
POG(16) p-t-octylphenol	Triton X-165			0.43
POG(17) cetyl-stearyl alcohol	Lubrol WX			0.02–0.06
POG(20) sorbitol mono-stearate	Tween 60			0.025
POG(20) sorbitol mono-oleate	Tween 80	60	50–76	0.012
POG(20) sorbitol mono-palmitate	Tween 40			0.027
POG(20) sorbitol mono-laurate	Tween 20			0.059
POG(20) cetyl alcohol	Brij 58			0.077
POG(29) oleyl alcohol	Brij 98			0.025
POG(40) p-t-octylphenol	Triton X-405			0.810
β-D-octylglucoside				25
Sodium cholate[3]		2–4	0.9–1.8	13–15
Sodium deoxycholate[3]		4–10	1.7–4.2	4–6
Sodium taurocholate[3]		4	2.2	10–15

[1]) Measured at 20–25°C
[2]) POG, polyoxyethyleneglycol
[3]) pH > 8; ionic strength < 10 mM

Several additional factors influence the choice of detergent:

1 *Material to be solubilized*
(a) An isolated membrane can be solubilized by most detergents. However, with whole nucleated cells it is desirable to keep the nucleus intact to avoid the problems associated with release of DNA. This can be accomplished by the use of some polyoxyethylene detergents (e.g. Nonidet P40, Triton X-100, Renex 30 — Table 4.3) under carefully controlled conditions (Section 4.4.2).
(b) Some proteins are not solubilized by certain detergents. A useful criterion for solubility is a high yield of the protein (measured for example by immunoassay, Chapter 11) in the supernatant after centrifugation at 100 000 ***g*** for 30 min.

2 *Detergent properties detrimental to subsequent procedures*
(a) Octyl and nonyl phenol detergents (e.g. Triton X-100 and Nonidet P40) have a high absorbance at 280 nm and hence interfere with protein monitoring (Section 1.1.1) during gel filtration or affinity chromatography. They also induce precipitation in the Folin protein assay (although the supernatant can be used to obtain values); they interfere less with the BCA modification and the Bradford assay (Sections 1.1.2 and 1.1.3).
(b) Octyl and nonyl phenol detergents are also easily iodinated and therefore should not be present during the radioiodination of proteins (Section 5.2). In addition, impurities of many other detergents can be iodinated (e.g. any unsaturated alkyl chain).
(c) Many detergents have a very high micellar weight (Table 4.4). This makes gel filtration impossible because the variation in protein size is usually insignificant compared with the size of the micelle. Also, removal of such detergents by dialysis (Section 1.3.1) is extremely slow because only the monomers can diffuse out of the dialysis sac, and the concentration of these (i.e. the critical micelle concentration) is usually very low (Table 4.4).
(d) The solubility of sodium cholate and deoxycholate decreases dramatically below pH 7.5 or above ionic strength 0.1.
(e) Ionic detergents, even sodium deoxycholate, interfere with electrophoresis and isoelectricfocusing (Chapters 6, 7 and 9).

Consideration of all these factors leads to the list of detergents recommended for various analytical procedures presented in Table 4.5. This is by no means comprehensive but should allow the reader to avoid some common pitfalls in initial experiments. Non-ionic detergents can be removed by absorption to beads in some circumstances (Drexler *et al.*, 1986).

4.4.2 Solubilization of whole lymphocytes

Under carefully controlled conditions, some non-ionic detergents

is usually less than 3 mm to avoid uneven heating effects. A gel size of 18 cm × 13 cm × 1.5 mm thick is suitable for most analytical purposes; this can accommodate up to 30 samples (see Section 9.2.1 for preparative polyacrylamide gel electrophoresis). Samples are loaded into slots formed in the stacking gel by inserting a Perspex comb into the gel solution before it polymerizes. Combs can be designed to accommodate the required number of samples. They should be cut from the same sheet of Perspex as the spacers and milled if necessary so that they are all the same thickness.

Apparatus for disc PAGE is best purchased (Shandon — Appendix 3). This technique differs from slab gel electrophoresis in that the gels are polymerized in tubes and hence are cylindrical in shape. The gels are often referred to as 'rods' or 'sticks'. The technique seems to have little or no advantage over slab gel electrophoresis (except for 2-dimensional electrophoresis, Section 7.6) and suffers from the disadvantage that each sample is electrophoresed in a separate gel making comparison of different samples difficult.

7.3 POLYACRYLAMIDE GEL ELECTROPHORESIS UNDER DENATURING CONDITIONS

The robust nature of polyacrylamide gel makes it an ideal support for electrophoresis in the presence of denaturing agents. Such techniques have the following advantages:

(a) Insoluble materials such as membranes and cytoskeletal structures can be solubilized and analysed in dissociating agents or detergents.

(b) Macromolecular complexes, such as enzymes and viral particles can be disrupted into their component polypeptides by a dissociating agent (especially if the disulphide bonds are broken) and then analysed by electrophoresis.

(c) If a strongly ionic detergent (usually sodium dodecyl sulphate — SDS; Tables 4.3 and 4.4) is used, then proteins are denatured and combine with a constant ratio (w/w) of SDS. The overwhelming negative charge provided by the SDS coating makes any charge contributed by the protein negligible, and so separation of such protein-SDS complexes by polyacrylamide gel electrophoresis is almost entirely due to sieving, and is therefore dependent on the molecular size of the protein. Thus a reasonably accurate value for the molecular weight of most proteins can be obtained by comparison of their electrophoretic mobility with those of standard proteins of known molecular weight. A high degree of glycosylation or gross deviation from 'normal' globular structure makes this determination inaccurate.

7.3.1 Sodium dodecyl sulphate (SDS) polacrylamide gel electrophoresis

This is probably the most widely used electrophoretic technique in heating with 2% SDS w/v plus a reducing agent (splits any disulphide bonds), and that the protein separation is usually dependent upon molecular weight (see Section 7.3).

Most proteins bind a constant amount of SDS (w/w) but there are exceptions to this which cause anomalous migration. For example, carbohydrate residues do not bind the detergent and so heavily glycosylated proteins migrate more slowly than non-glycosylated proteins of the same molecular weight. Also some proteins (e.g. J chain) are not fully unfolded by the SDS treatment and so migrate more slowly than expected (Fig. 7.4).

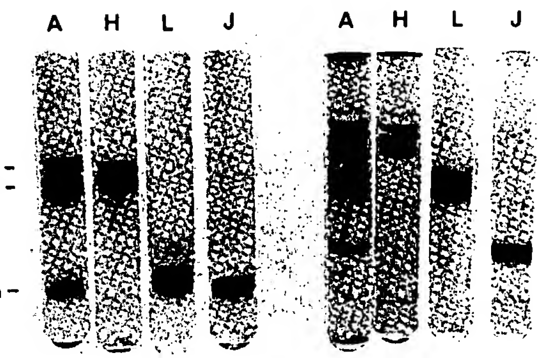


Fig. 7.4 Disc polyacrylamide gel electrophoresis in the presence of urea (right) or SDS (left) (Sections 7.3.1 and 7.3.3). A, reduced and alkylated rabbit secretory IgA; H, and J, fractions from the separation of reduced and alkylated secretory IgA (see Fig. 3.11). Note that J chain is only resolved from L chain by the urea gel. This gel (separating proteins on the basis of charge rather than size) also demonstrates the heterogeneity of the α and L chains (due to their variable regions) compared with J chain and secretory component.

Many buffer systems have been developed for SDS-polyacrylamide gel electrophoresis, and two of these are described in this section. Method i is Laemmli's modification of the Ornstein-Davis buffer system described in Section 7.2. Method ii is simpler and covers a wider molecular weight range. Examples of SDS-polyacrylamide gel electrophoresis of standard proteins using the two systems are shown in Fig. 7.5.

Method i (Laemmli, 1970)

Materials and equipment

Power pack (Shandon, LKB — Appendix 3)

example, see Fig. 7.8). Proteins undergo increasing molecular sieving as they migrate through the gel and this allows analysis of proteins over a wide molecular weight range. 5–15% (w/v) acrylamide gradients are suitable for most purposes.

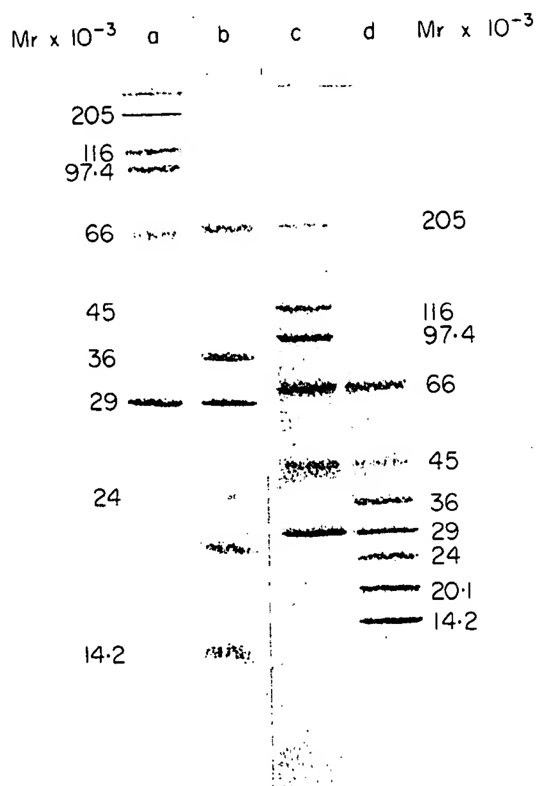


Fig. 7.8 SDS–polyacrylamide gel electrophoresis of commercially available molecular weight marker proteins (Sigma — Appendix 3). Tracks a and c high Mr range, b and d low Mr range. Tracks a and b analysed using a 12.5% total acrylamide slab gel (Section 7.3.1, method i); (c) and (d) analysed using a 5–15% total acrylamide linear gradient gel (Section 7.3.2). The gradient gel shows a compaction of the molecular weight range and produces tighter bands, particularly in the low Mr range.

Materials and equipment

As for Section 7.3.1, method i
2 chambered linear gradient maker (Section 1.2.2; Fig. 1.3) or better a 3 channel pump (Shuco — Appendix 3)
Sucrose
Magnetic stirrer

Procedure

The procedure differs from Section 7.3.1 only in the preparation of

1 Prepare heavy gel solution (15% w/v total acrylamide). For this add 4.5 g sucrose to 11.25 ml 1 M tris-HCl pH 8.8, 9.0 ml stock acrylamide, 0.7 ml ammonium persulphate solution and 0.3 ml 10% w/v SDS solution and make up to 30 ml with water. Mix thoroughly until all the sucrose dissolves. Use 10 ml of this solution to seal the gel trough (if required) — see Section 7.3.1, step 2.
2 Prepare light gel solution (5% w/v total acrylamide). For this mix 7.5 ml tris-HCl pH 8.8 with 2 ml stock acrylamide, 0.47 ml ammonium persulphate solution, 0.2 ml 10% w/v SDS solution and make up to 20 ml with water.
3 Add 5 μl TEMED to each gel solution mix and pipette 12 ml (see note 1) of each into separate chambers of the gradient maker. Stir the heavy solution and prepare the gradient (Fig. 1.3) by pumping from the heavy side slowly into the top of the gel mould (should take about 5–10 min). Alternatively, if a 3 channel pump is available, place the solutions in 25 ml beakers and stir the heavy one. Use *two* channels to pump from the heavy solution to the gel mould and *one* channel to pump light solution into the heavy solution. All channels must pump at the same rate.
4 Overlay with isobutanol saturated with water and leave to polymerize.
5 Prepare the stacking gel (Section 7.3.1, method i, step 5) and continue as for Section 7.3.1, method i, steps 6–11.

Notes

1 For apparatus different from that shown in Fig. 7.3 use equal volumes of heavy and light solutions to give the total volume required to fill the mould to 3.0–4.0 cm from the top.
2 If gradients other than 5–15% are required, adjust the light and heavy solutions to give initial and final acrylamide concentrations respectively.

7.3.3 Urea polyacrylamide gel electrophoresis

Most 'insoluble' proteins can be solubilized and electrophoresed in urea. Proteins which consist of disulphide-linked subunits can be split into constituent chains by reduction with dithiothreitol.

Materials and equipment

As for Section 7.3.1 but substitute solutions:
Stock acrylamide solution — 7% (w/v) acrylamide, 0.35% (w/v) N,N'-methylene bisacrylamide, 45% (w/v) urea (7.5 M) made up to

TEMED

Electrode buffer — 0.05 M boric acid adjusted to pH 9.2 with sodium hydroxide (700 ml per run)

Sample buffer —

with reduction: 8 M urea containing 2% w/v dithiothreitol

without reduction: 8 M urea containing 0.004 M iodoacetamide prepared with 0.37 M tris-HCl pH 8.8

Procedure

1 Assemble gel mould (Section 7.3.1, steps 1 and 2).

2 Prepare gel solution by mixing 32 ml of stock acrylamide solution, 7.2 ml of water and 0.8 ml of ammonium persulphate solution and degas under vacuum. Add 10 μl TEMED, mix and completely fill the mould with gel solution. Insert plastic comb into the top of the gel to form slots and leave to polymerize.

3 Remove comb carefully, wash slots with electrode buffer and clamp gel to electrophoresis tank (Secion 7.3.1, step 6). Fill reservoirs with electrode buffer.

4 Prepare samples for electrophoresis by mixing with an equal volume of sample buffer and boiling for 1 min. Load samples onto gel as described in Section 7.3.1, step 9, and electrophorese (Section 7.3.1, step 10) for 2–3 h at 10 V/cm length of gel.

5 Turn off power, remove gel from mould and stain (Section 7.1.1 and 7.1.2).

Notes

1 If both reduced and unreduced samples are electrophoresed in the same slab, add 1/10 volume of 1.5 M iodoacetate to the reduced samples after boiling to destroy excess reducing agent.

2 Bromophenol blue (0.02%) can be added to the sample buffer to monitor the progress of the electrophoresis. The free dye migrates faster than any protein. If albumin (normally one of the faster moving proteins) is present, some of the dye will bind to this and show its position. To separate slow moving proteins, continue electrophoresis for 1–2 h after the dye has run off the bottom of the gel.

3 Quantity of sample to be loaded depends on protein concentration, heterogeneity of the sample and the ability of the individual proteins to bind stain — see Section 7.1.1 and 7.1.2.

4 If heating effects cause distortion of bands, cool the apparatus with a fan (carrying out electrophoresis at 4–8°C might precipitate the urea).

5 See note 8, Section 7.3.1.

7.3.4 Disc PAGE under denaturing conditions

All of the procedures in Sections 7.3.1 to 7.3.3 can be carried out in polyacrylamide rods using disc gel apparatus and adjusting the buff[...] volume appropriately.

Materials and equipment

As for slab polyacrylamide gel electrophoresis (Section 7.3.1) excep[t] an electrophoresis tank suitable for disc gel electrophoresis is require[d] (Shandon — Appendix 3).

Glass tubes, about 10 cm × 0.6 cm (lab-made or Shandon — Appendix 3)

Rubber bungs for sealing the bottoms of the tubes

10 ml syringe with long hypodermic needle

Procedure

1 Seal the base of the glass tubes with rubber bungs (see no[te] and layer of parafilm (or use commercially available 'sealing bungs'). Plac[e] sealed tubes *vertically* in a test tube rack.

2 Prepare separating gel solution (Section 7.3.1, step 3); 2 ml i[s] required per rod. Pipette gel solution into each mould leaving a 3 c[m] gap at the top of each tube. Overlay with isobutanol and leave t[o] polymerize (see Section 7.3.1, step 4).

3 Just before electrophoresis is to be carried out prepare the stackin[g] gel solution (0.3 ml per gel rod — see Section 7.3.1, step 5). Wash th[e] isobutanol from the gel surface and overlay the separating gel wit[h] stacking gel solution (1.0–1.5 cm high). Overlay *carefully* with isobutanol and leave to polymerize (10–20 min).

4 Rinse off the isobutanol from the stacking gel surface and positio[n] the rods in the electrophoresis tank. Fill electrode reservoirs wit[h] electrode buffer (Section 7.3.1, step 7) and remove any air bubbl[es] lodged at the top and bottom of the gels by displacement with electrode buffer forced from the syringe.

5 Load samples (Section 7.3.1, step 9 and note 3) onto the surface [of] the gels, connect the tank to the power pack (lower compartme[nt] positive) and electrophorese at 1.5 mA/tube for 20 min followed b[y] 3.0–3.5 h at 2 mA/tube.

6 Turn off power and remove rods from the tank. Remove gels fro[m] the glass tubes by 'rimming' with the hypodermic syringe and needl[e]. Fill the syringe with water and insert the needle in between the gel an[d] the glass tube. Hold the tube over a test tube and rotate it whil[e] expelling water from the syringe. The gel will fall into the test tub[e]. Stain and destain the gel (Sections 7.1.1 or 7.1.2).

Note

The glass tubes should be soaked in chromic acid (Appendix 2) an[d] rinsed and dried thoroughly before use.

7.4 POLYACRYLAMIDE GEL ELECTROPHORESIS UNDER NON-DENATURING CONDITIONS

In non-denaturing PAGE, proteins in the sample are neither disrupted nor denatured, and migration is largely due to the inherent charge of the components. Usually low concentrations of polyacrylamide are used (4–8%) because the molecular sieving effect of higher concentrations effects the charge-dependent separation. As the proteins are electrophoresed unaltered, the technique is sometimes called native PAGE. Many different buffer systems have been described for this technique, but many lack resolution or are only suitable for a particular class of proteins.

Probably the most widely used native PAGE system is that described by Ornstein (1964) and this may be carried out as described for SDS-PAGE in Section 7.3.1, method i, by omitting SDS from all solutions. The samples should be dialysed against electrode buffer and then sucrose (final 10% w/v) and bromophenol blue (final 0.005%) added before loading; they should not be heated. The stock electrode buffer should be diluted 40- or 80-fold rather than the 10-fold used for SDS-PAGE. Carry out electrophoresis at 4–8°C to avoid uneven heating effects.

Native disc PAGE can be carried out using the same modifications to Section 7.3.4.

7.5 ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS

7.5.1 Introduction

This technique is capable of the greatest resolution of a protein mixture. The components are separated according to their isoelectric point (pI) which is the pH at which they possess no net electric charge.

A pH gradient is formed in a gel between cathode and anode, and proteins migrate (due to the attraction of their charged groups for anode or cathode) until they reach their pI. As the protein is not charged in this position it remains stationary. Isoelectric focusing is therefore a steady state process (components should theoretically never run off the end of a gel containing the correct pH gradient).

A pH gradient will form automatically if a potential difference is applied to an H^+ containing ionizable solvent (H^+ ions will be displaced towards the cathode). However, such gradients are unstable and so gradient stabilizing molecules are added. These are normally purpose-made amphoteric compounds known as 'carrier ampholytes'. These zwitterions have sharply defined pIs and the mixture is chosen to have pI values evenly distributed over the desired pH range. Such mixtures are commercially available.

Polyacrylamide gel is an ideal anticonvention medium for isoelectric prepare and shows very little electroendosmosis (Section 6.4.1). This latter property is very important in isoelectric focusing as electroendosmosis will cause the whole pH gradient to move toward the cathode and thus disrupt the steady state process. However, polyacrylamide is not sufficiently permeable to very large proteins (e.g. pentameric IgM, Section 3.5) and special agarose is available for isoelectric focusing of such proteins (LKB, Pharmacia — Appendix 3).

7.5.2 Focusing in slab gels

Isoelectric focusing can be carried out in rods or slabs of polyacrylamide. Slabs are usually preferred, as several samples can be compared on one gel and uneven heating effects can be avoided more easily. Samples can be applied to the gel away from the strong acid or alkaline electrode solutions, hence avoiding denaturation of the protein components. It is advisable to use riboflavin as the initiator of polymerization rather than ammonium persulphate, because the latter reagent oxidizes proteins and ampholytes and can lead to artefactual results.

All the equipment necessary for isoelectric focusing in polyacrylamide gel including electrophoresis chamber, cooling plate, power pack and even ready-made ampholyte containing polyacrylamide gels are available commercially (LKB, Pharmacia — Appendix 3). Such set-ups are expensive but it is usually impractical to synthesize one's own ampholytes and a high voltage power pack and complex chamber with safety cut-outs are necessary.

Materials and equipment

Ampholytes (LKB, Pharmacia — Appendix 3)
High voltage (preferably constant power) power pack (LKB, Pharmacia — Appendix 3)
Commercially available gel mould, or lab-made mould consisting of 2 glass plates 15 × 10 cm, 2 Perspex spacers 16 cm long × 1 cm wide × 0.1 cm thick and 4 bulldog clips — see Fig. 7.3, (a) and (b)
Petroleum jelly (e.g. Vaseline)
Electrophoresis tank suitable for isoelectric focusing equipped with platinum electrodes (LKB, Pharmacia — Appendix 3) (see note 1)
Riboflavin solution: 4 mg/100 ml water freshly prepared
Stock acrylamide solution: 28.5% (w/v) acrylamide, 1.5% (w/v) N,N′-methylene bisacrylamide (store at 4°C — see note 2)
TEMED
UV lamp (wavelength of emitted light = 350 nm) or fluorescent strip light
Electrode solution (Table 7.3)
Whatman 3 MM filter paper
Fixing solution: 3.5% (w/v) sulphosalicylic acid, 11.5% (w/v) trichloroacetic acid